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Research Paper

In vitro determination of genotoxic effects of sodium benzoate preservative on human peripheral blood lymphocytes

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Abstract

Sodium Benzoate E211 was food preservatives which inhibits the microbial growth. The Genotoxic effect of sodium benzoate on human lymphocytes was studied by the using chromosomal aberration and sister chromatid exchanges assay. Lymphocytes were treated with three different concentration of sodium benzoate which was 0.5, 1.0, and 1.5 mg/ml. Sodium benzoate was significantly increase the level of chromosomal aberration and sister chromatid exchanges with compare to control (*P* < 0.05). Sodium benzoate was also decrease the index of cell cycle proliferation which can leads to cell cycle delay. Six types of structural aberration were observed which were as follows, Chromatid gap and breaks, Chromosome gap and break, Acentric fragment and Dicentric chromosome. Chromatid gaps were most frequent (75.62%) in all of this structural aberration. Sodium benzoate is Genotoxic for lymphocytes which can induce genomic instability.

Keywords: Genotoxicity, Food Preservatives, Human Lymphocytes, Sodium Benzoate, Chromosomal Aberration, Sister Chromatid Exchanges etc.

Abbreviations

FDA	Food and Drug Administration
CAs	Chromosomal Aberrations
SCEs	Sister Chromatid Exchanges
MN	Micronucleus
SB	Sodium Benzoate
PB	Potassium Benzoate
CCPI	Cell Cycle Proliferation Index

Introduction

Food Preservatives are one of the commonest additives which can be used to prevent microbial growth and other chemical changes in food. For example Preservatives including benzoate and Sorbate groups are used as fungistatic and bacteriostatic in food which has acidic in nature like carbonated drinks, fruit juice, condiments etc[1].

Food safety cannot be a newer concept of the modern era, it can be seen in all over the history of human civilization. In most of the developed countries, the problems were raised after the 2nd world war. Food spoilage has been a common problem and it can occur mostly due to activities of microbes. Food preservation methods have been common both natural and chemical since the past 1000 to 8000 years. In early era natural preservatives like salts, sugars, and alcohol were used[2].

From last few years uses of food additives were enormously increased, about 75% of the western diet made up of processed foods, therefore each person consume about 8-10 Ibs of food additives every

year. Food additives have some adverse effects like, urticaria, angioedema, eczema, exfoliative dermatitis, nausea, irritable bowel syndrome, vomiting, nausea, diarrhoea, rhinitis, migraine, anaphylaxis, bronchospasm, hyperactivity and other behavioural disorders[3]. Food preservatives have adverse effects on human body organs like liver and kidney, they also have the effects on individual cell and cell organelles. Food preservatives lead chromosomal breakage, which can be studied by different parameters like Chromosomal aberration (CA) study, Sister Chromatid Exchange (SCE) test, Micronucleus (MN) test, and Comet assay. For these studies mainly Lymphocytes were used. Which can be cultured by the method of Hungerford[4].

Sodium Benzoate [E211], Potassium Benzoate [E212], Sodium Sorbate [E201], Potassium Sorbate [E202] are most commonly used preservatives. Which can be used in Beverages like carbonated and non-carbonated, Cider, Margarine, syrup, Fruit juices, Fruit jam, Fruit butter, Pickles, and in some medicines.[5]. FDA approved different concentrations of these preservatives, Benzoic acid used upto 0.1%, Sodium benzoate less than 0.1%, Sodium Sorbate less than 0.3%, Potassium Sorbate less than 0.3%, Potassium benzoate upto 0.1%, and Sorbic Acid less than 0.2%.

Sodium Benzoate is synthetic additive which is the salts of benzoic acid. Sodium benzoate has used as preservatives in food industry for protect the food from bacteria, yeast and Fungi, it can be used at pH 4.5. It can also been used in pharmaceutical and cosmetic industries[6]. In 1999, researcher can prove that the sodium salt of benzoic acid has not the bactericidal but only bacteriostatic with fungi static activity, it can be activated only in acidic condition[7].

The uses of Chemical Preservatives can be started in the 19th century. In the beginning of 20th century in 1908, Sodium benzoate can officially sanction for used as preservatives by the United States[2]. This all preservatives have some or more hazardous effects on chromosomes. For that many *In vitro* studies can be carried out by researchers.

In 2008, P.Mpountoukas, A.Vantarakis, E.Sivridis, T.Lialiaris were studied the Genotoxic effect of three commonly used preservatives Sodium benzoate, Potassium Sorbate, Potassium nitrate on human lymphocytes[8]. They were carried out Sister Chromatid exchange (SCEs) assay for that they were used Fluorescence plus Giemsa (FPG) Technique[9]. They were used the different concentration 0.02, 0.2, 2, 4, 8 mM of Sodium benzoate, Potassium Sorbate, Potassium nitrate. They were evaluated the statistical data analysis by ANOVA procedure and use Duncan test for pair-wise comparisons[10,11].

In 2010, Researchers carried out Genotoxic studies of two food preservatives Sodium Benzoate, and Potassium Benzoate[12]. They were used three assay for their study, Chromosomal aberration, Sister chromatid exchanges and Micronucleus assay. For CAs and SCEs test they were used the methods of Evans (1984) and Perry & Thompson (1984), with some modifications according to Yüzbaşioğlu's method[13]. For SCEs assay chromosomes were stained with Giemsa by Speit and Houpter's method[14] with some modifications according to Mamur's method[15]. Preparation of Micronucleus was done according to the method of Fenech and Palus [18] and the Comet assay was performed according to the method of Singh et al's. [17]. They can used different concentration of SB and PB. They were used 6.25, 12.5, 25, 50, and 100 µg/ml concentrations of SB, and 62.5, 125, 250, 500, and 1000 µg/ml concentration of PB.

In 2015, Researcher carried out a study to know the effects of Sodium Benzoate preservative in Human lymphocytes[1]. Four different concentration of Sodium Benzoate were used 0.5, 1.0, 1.5, & 2.0 mg/ml and two treatments were given, of 24 hr and 48 hr. Micronucleus test performed as per Fenech and Palus. For Chromosomal aberration study, Chromosomes were stained by Giemsa as per the method of Seabright[19].

Materials and Method

This *In vitro* Genotoxic study of sodium benzoate was carried out on Human Lymphocytes. For that venous blood collected from Normal healthy individuals of the age group of 20-30 years were selected for the blood sample. Each person was not having any type of infection and was not having any addiction of tobacco, alcohol and smoking.

Three different doses of Sodium Benzoate were prepared in sterile core distilled water and filtered it through 0.22 µm filter. Final dose concentration was 0.5, 1.0, and 1.5 mg/ml. For the lymphocytes

culture, standard method of Hungerford was used[4]. 7 ml of RPMI 1640 media and 0.5 ml of heparinised blood were placed in culture tube. RPMI 1640 media was presupplemented with 10% FBS, 0.1 ml of PHA and Penicillin/ Streptomycin. Different doses of sodium benzoate were also added at the beginning. The culture tubes were placed in CO_2 incubator at 37ºC for 72 hours. At 69th hour 20 µl of Colchicine was added and then culture tube placed again in CO_2 incubator at 37ºC for one hour. After that tubes were centrifuged at 2000 rpm for 15 min. Supernatant discarded, Then 5 ml of Hypotonic solution 0.56% KCl were added into tubes and incubate the tubes in water bath at 37ºC for 30 min. Fixative 1:3 Acetic acid: methanol were added into tube after 30 min. the centrifuged at 2000 rpm for 15 min. Washes of fixative were given till the pellet become clear. After that slides were prepared. For Sister Chromatid exchanges 80 µl of BrdU was added at the beginning or at 0th hour.

Slides were directly stained with Giemsa for chromosomal aberration study and for sister chromatid exchanges assay, slides were stained by the Fluorescence plus Giemsa staining method of Perry and Wolf[20]. 200 well separated metaphase plate were scored for chromosomal aberration and cell cycle proliferation index and 50 M_2 metaphase plate were scored for sister chromatid exchanges. t-test was used for the statistical data analysis.

Results and Discussion

Chromosomal Aberration

Sodium benzoate can induce a significant increase in the frequency of CAs and CAs/cell in all concentrations compare to control. Sodium benzoate can causes six types of structural aberration in chromosomes which are as follow, chromatid gaps, chromatid breaks, chromosome gaps, chromosome breaks, acentric fragments and dicentric chromosomes. Chromatid gaps (75.62%) and chromatid breaks (19.57%) are the most frequent aberrations in all of six types. Other results of chromosomal aberration analysis are shown in table 1.

Table 1: Results of Chromosomal Aberration analysis

Test Substance	Concentration (mg/ml)	Aberration						CAs/plate ± SE
		ctg	ctb	csg	csb	af	dic	
Control	0.0	6	1	-	-	-	-	0.035 ± 0.005
Sodium	0.5	26	8	-	-	1	-	0.173 ± 0.007*
Benzoate	1.0	54	13	1	1	3	-	0.363 ± 0.013*
	1.5	87	24	1	2	4	3	0.605 ± 0.015*
Freq. of Aberration(%)		75.62	19.57	0.85	1.28	3.40	1.28	

ctg: chromatid gap, ctb: chromatid break, csg: chromosome gap, csb: chromosome break, af: acentric fragment, dic: dicentric chromosome.
Total 200 plates are scored for each treatment.
* Significant from the control $P < 0.05$ (t – test).

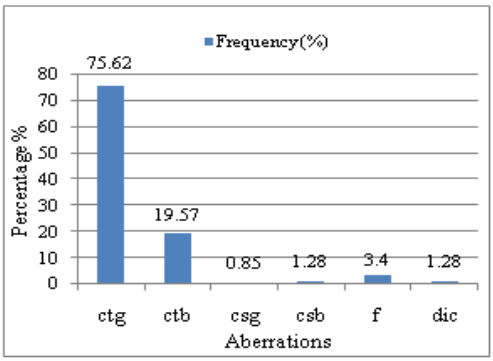

Figure 1: Graphical representation of percentage of frequency of six different types of chromosomal aberration

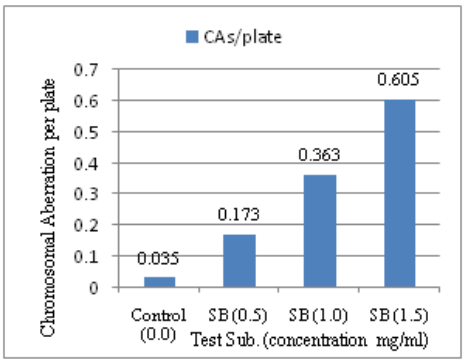

Figure 2: Graphical representation of Chromosomal Aberration per plate of control and different doses of sodium benzoate

Sister Chromatid Exchanges

The result of sister chromatid exchanges analysis are shown in table 2. Sodium benzoate increase the frequency of sister chromatid exchanges per cell. This increase is significant in all different concentrations of sodium benzoate. The increase of sister chromatid exchanges is concentration dependent.

Table 2: Result of Sister Chromatid exchanges analysis

Test Substance	Conc. (mg/ml)	Min – Max SCEs	SCEs/plate ± SE
Control	0.0	0 – 5	3.640 ± 0.040
	0.5	4 – 18	9.870 ± 0.270*
Sodium Benzoate	1.0	4 – 20	12.360 ± 0.160*
	1.5	5 – 27	15.310 ± 0.270*

50 M_2 Metaphase plates are scored for each treatment.
* Significant from the control $P < 0.05$ (t – test)

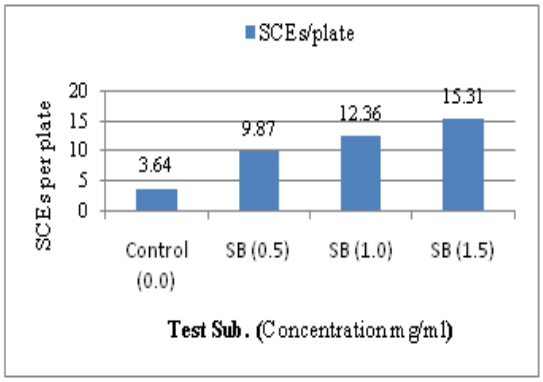

Figure 3: Graphical representation of sister chromatid exchanges per plate of control and different doses of sodium benzoate

Cell Cycle Proliferation Index

The result of cell cycle proliferation index is shown in table 3. The value of cell cycle proliferation index is significantly decreased when the concentration of sodium benzoate is increase.

Table 3: Result of cell cycle proliferation index analysis

Test Substance	Conc. (mg/ml)	M_1	M_2	M_3	CCPI ± SE
Control	0.0	39	63	98	2.275 ± 0.015
	0.5	46	67	87	2.175 ± 0.025
Sodium Benzoate	1.0	59	79	62	2.030 ± 0.020
	1.5	67	75	58	1.930 ± 0.030

Total 200 plates are scored for each treatment

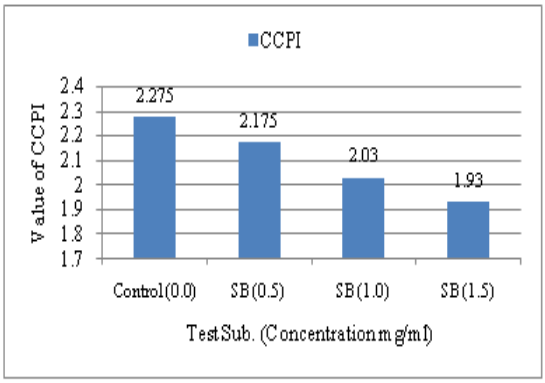

Figure 4: Graphical representation of cell cycle proliferation index of control and different doses of sodium benzoate

In this *In vitro* study, the Genotoxic potential of Sodium Benzoate is investigated, with chromosomal aberration, sister chromatid exchanges, and cell cycle proliferation index analysis in the cultured human peripheral lymphocytes. *In vitro* genotoxicity tests detected substances that can induce genetic damage, directly or indirectly, by different mechanism. That all substances are considered to be the markers of early biological effects of carcinogenic exposure[21].

Sodium benzoate can be significantly increased the frequency of chromosomal aberrations and chromosomal aberration per plate in all treatment when compared with control. Sodium benzoate can be induced six types of structural aberrations (Chromatid gap, chromatid break, chromosome gap, chromatid break, dicentric chromosomes and acentric fragment), which can be indicated their clastogenic effects.

In this *In vitro* study, chromatid gaps and chromatid breaks have been observed as a most common type of aberrations. This types of chromosomal breakage can be resulted in a number of different structural rearrangements, some of which gave rise to the abnormalities of chromosomal segregation at mitosis[22]. Increased levels of the chromosomal aberration can be associated with increased risk of cancer[22].

Sodium benzoate can also increase the frequency of Sister chromatid exchanges per plate in all concentration compare with control. High frequency of sister chromatid exchanges can be observed in the persons at higher cancer risk, which can be occurred due to occupational or environmental exposure to a wide variety of carcinogens[23–25]. Sodium benzoate can also decrease the cell cycle proliferation index. It can be occurred due to blockage of the activity of the mitogen, which can trigger the cell division.

Sasaki et al. reported that sodium benzoate cannot be yield a statistically significant increase in DNA damage in any of the mouse organs. They were used about 2000 mg/kg amount of sodium benzoate as a dose[26]. On the other side, the sister chromatid exchanges test were carried out on the *V. faba* root tip cells and human peripheral blood lymphocytes, at a dose level of 0.02M, which shows the significant increase of sister chromatid exchanges per plate in comparison to the control[27]. Turkoglu reported that sodium benzoate can significantly increase the chromosomal aberration and decrease the mitotic index in *A. cepa*[28]. In fibroblast cell line of Chinese hamster, sodium benzoate can also show positive results of chromosomal aberration test and the sister chromatid exchanges test[29,30].

In some of the recent studies, reported that the sodium benzoate can significantly increase the level of sister chromatid exchanges at the 2 mM, 4 mM and 8 mM dose concentration. This study carried out on the human peripheral blood lymphocyte. They can also observed the cell cycle delay in compare with control[8].

In 2011, also reported that different concentration of sodium benzoate can significantly increase the level of chromosomal aberration and the sister chromatid exchanges in compare to the control in both 24 hr. and 48 hr. of incubation with dose. This study was carried out on the human peripheral blood lymphocytes. In this study, chromatid break and chromosomal break were observed in higher frequency. Mitotic index and cell cycle proliferation index were decreased with the increasing concentration[12]. In 2015, also reported that sodium benzoate can caused sister chromatid separation and chromosomal gaps at higher concentration in compare to control. This study was also carried out on the human peripheral blood lymphocytes[1]. Among this all study most of study can reported that the sodium benzoate can significantly increase the level of chromosomal aberration and sister chromatid exchanges, and decrease the rate of cell cycle proliferation index which was significantly matched with my results.

Conclusion

From this *In vitro* Genotoxic study, it can be concluded that the sodium benzoate can significantly induce chromosomal aberration at higher concentration and Sister chromatid exchanges in almost all concentration compare to the control. Sodium benzoate can also decrease the cell cycle proliferation index in all concentration compare to the control. The results observed in this study can obtain the progress knowledge to prevent diseases caused by genomic instability or decrease the numerical and structural changes of human chromosomes caused by consumption of food additives and insist on more extensive safety assessment of food preservatives to activate the government departments working for public health to be concerned about disadvantages of food additives.

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